



EXHIBIT D2

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CHEMICAL AND BIO-SENSORS

THE APPLICATION OF CYTOCHROMES AS THE INTERFACE MOLECULE TO FACILITATE THE ELECTRON TRANSFER FOR PQQ GLUCOSE DEHYDROGENASE EMPLOYING MEDIATOR TYPE GLUCOSE SENSOR

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ABSTRACT

In order to improve the sensor response of mediator-type glucose enzyme electrode, we focused on the application of the electron transfer proteins, cytochromes, as interface molecules to facilitate the electron transfer from enzyme to artificial electron mediator. In this paper we used cytochrome *c* and cytochrome *b₅₆₂* for the improvement of sensor signal of glucose enzyme sensor employing glucose dehydrogenase harboring pyrroloquinoline quinone (PQQGDH). When sensors were operated using either potassium ferricyanide or 1-methoxy-5-methylphenazinium methylsulfate (mPMS) as the artificial electron mediator, the response was over

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30-fold greater with the co-immobilization of either cytochrome *c* or cytochrome *b₅₆₂* than with PQQGDH alone. The impact of the cytochrome co-immobilization was dependent on the amount of cytochromes, indicating that these cytochromes facilitated the electron transfer from the PQQGDH redox center to the artificial electron mediators used in the sensor system. These results demonstrated the future application of cytochromes as an essential component for the improvement of sensor response in the redox enzyme-based amperometric sensors.

INTRODUCTION

Self care blood glucose monitoring systems have conventionally been developed based on glucose oxidase (GOD) since the discovery of the principle of enzyme sensor.^[1,2] However, the recent trend of glucose sensor development now focuses on the mediator-type glucose sensor, in which the effect of dissolved oxygen toward the inherent property of GOD is significant. Much attention is therefore given to glucose dehydrogenases harboring pyrroloquinoline quinone (PQQGDHs) as the ideal glucose sensor constituent as these enzymes cannot utilize oxygen as the electron acceptor during the oxidation of glucose.^[3,4] Two types of PQQGDHs have been reported, the membrane-bound monomeric PQQGDH-A and the water soluble dimeric PQQGDH-B.^[5,6] Although both PQQGDHs are only negligibly affected by the presence of the oxygen, their stabilities and substrate specificities should be further improved compared with those of GOD. The authors have therefore been carrying out protein engineering studies in order to improve the enzymatic properties of both PQQGDHs.^[7-18] Considering the easy handling and also the high catalytic efficiency, the requirement for the water soluble PQQGDH (PQQGDH-B) is increasing. PQQGDH-B has only been reported from the periplasmic space of the Gram negative bacterium *Acinetobacter calcoaceticus*, however, the physiological role of PQQGDH-B remains unknown.

Several mediator type glucose sensors utilizing PQQGDH-B have been reported.^[19,20] Due to the preference of this enzyme toward the artificial electron mediator,^[21] the sensor signal is greatly dependent on the type of mediator employed in each sensor system (Fig. 1). For example, PQQGDH-B shows high catalytic current when phenazine methosulphate (PMS) or Ruthenium complex were utilized as the mediator. However, PQQGDH-B does not efficiently utilize potassium ferricyanide, a stable, cost effective mediator used in a large number of electrochemical sensors.



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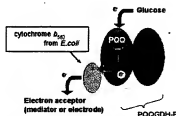


Figure 1. The concept of this study.

The further improvement of mediator preference of PQQGDH-B-based mediator-type enzyme sensors is therefore also protein target of the engineering.

In order to improve mediator dependent sensor signal property, we focused on the application of electron transfer proteins, cytochromes. In nature, various dehydrogenases harboring either PQQ or FAD are coupled with the respiratory chain via cytochromes. Based on this electron transfer property, we considered the utilization of cytochrome as the interface molecule to facilitate the electron transfer between PQQGDH and potassium ferricyanide in the GDH sensor. In this study, we used two different cytochromes, cytochrome *c* from horse heart and cytochrome *b*₅₆₂ from *Escherichia coli*. Cytochrome *c* has already been reported as the electron transfer subunit of several PQQ/FAD-dependent dehydrogenases. There are also some reports of cytochrome *c* as the endogenous electron acceptor for enzyme electrochemistry.^[22,23]

E. coli cytochrome *b*₅₆₂ is a monomeric 12 kDa protein molecular mass^[24] localized in the periplasm,^[25] but its physiological function is unknown. The protein adopts a four-helical bundle fold and coordinates to the heme iron through ligands provided by methionine 7 and histidine 102, residues that are close to the termini of the polypeptide. It is assumed to be a simple electron carrier shuttling electron between other (membrane bound) redox centers, but no natural partners have been identified. In contrast to many other simple electron carrier proteins, it is not highly charged and does not display obvious areas of surface charge with which it might bind electrostatically to partner redox proteins. There is, however, a cluster of acidic groups (including the heme propionates) around the *N*-terminal region of the protein. The reduction potential of the wild type has been measured by potentiometric techniques.^[24,26] The ease of overproduction in *E. coli*^[25,27] and availability of high resolution structure of both holo- and apo-proteins^[28,29] make this cytochrome an excellent starting point for



the generation and study of cytochromes with novel redox and electron transfer properties.

EXPERIMENTAL SECTION

Chemicals

Cytochrome *c* from horse heart was purchased from Sigma-Aldrich (St. Louis, USA). Potassium ferricyanide, 1-methoxy-5-methylphenazinium methylsulfate (mPMS) were purchased from WAKO (Osaka, Japan).

DNA Manipulation

The structural gene of cytochrome *b₅₆₂* was cloned from *E. coli* B strain into the high expression vector plasmid pTrc99A (Pharmacia Biotech.) (Fig. 2). The published sequence^[25,30] of *cybC*, the structural gene of cytochrome *b₅₆₂*, was used to design forward and reverse oligonucleotide primers (*Nco*I and *Bam*HI, respectively) to be used to amplify the entire gene, including the signal peptide, with appropriate restriction sites (*Nco*I and *Bam*HI) at each terminus. The oligonucleotides used in this study are the following:

*Nco*I: 5'-GGGGGCCATGGGGCGTAAAAGCCTGTTAGCTATT-CTTGCACTCTCC-3',

*Bam*HI: 5'-GGGGGGGATCCTTAACGATACTTCTGGTGATAGG-CGTTGCGGG-3'.

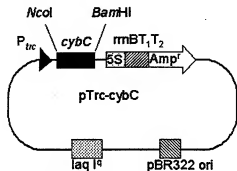


Figure 2. Expression vector for cytochrome *b₅₆₂*, pTrc-*cybC*.



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The creation of a *Nco*I site at the initiating methionine codon required the insertion of a GGG (glycine) codon at the start of the signal sequence. The gene was amplified from a genomic DNA preparation of *E. coli* B cells using these primers according to standard PCR technique. *Taq* polymerase was used in a 30-cycle amplification with annealing at 50°C for 1 min and extension at 72°C for 1 min. The PCR product was purified by agarose gel electrophoresis before digestion with both *Nco*I and *Bam*HI restriction enzymes. Digested PCR product was ligated with the gel-purified 4.2 kDa fragment of *Nco*I–*Bam*HI digested pTrc99A plasmid. The ligation mix was transformed into *E. coli* strain DH5 α . Cytochrome *b*₅₆₂ expressing cells were selected by their pink color. The expression vector was purified, and the sequence was confirmed by standard dideoxynucleotide sequencing technique. Thus constructed plasmid for the expression of *E. coli* cytochrome *b*₅₆₂ was designated as pTrc-cybC.

Preparation of Recombinant Cytochrome *b*₅₆₂ and PQQGDH-B

Recombinant cytochrome *b*₅₆₂ was expressed in *E. coli* strain DH5 α harboring pTrc-cybC cultured in 2 L of L media at 37°C. 300 μ M IPTG was added at OD₆₀₀ = 1, and the incubation was continued for 18 h after induction. *E. coli* cells were harvested by centrifugation, and periplasmic fraction was obtained by freeze-thaw lysis using 10 mM MOPS buffer, pH 7.2. The material was absorbed onto DEAE-5pw column (TOHSON), and eluted with 200 mM NaCl in the same buffer. Eluted protein was separated by a size exclusion column (Hiroad column, Pharmacia), and purified protein was evaluated by SDS-PAGE.

Recombinant PQQGDH-B was expressed in *E. coli* using PQQGDH-B structural gene from *Acinetobacter calcoaceticus* inserted in the expression vector pTrc99A, and the purified enzyme was prepared according to our previous report.^[12]

Preparation of PQQGDH/B-Cytochrome
Co-immobilized Electrode

Ten mM MOPS buffer (pH 7.0) containing 25 U (0.67×10^{-10} mol) of PQQGDH-B and appropriate concentration of cytochrome (appropriate fold molar excess of cytochrome *b*₅₆₂ or cytochrome *c*) was mixed with carbon paste (0.5 g graphite powder mixed with 0.3 mL paraffin liquid) and lyophilized. The lyophilized mixture was then packed into the end of the carbon electrode (3 mm i.d., BAS Inc, West Lafayette, USA) and fixed



with 1% glutaraldehyde solution for 30 min and washed with 10 mM *tris*-HCl buffer (pH 7.0). The electrode was then allowed the holo-formation in 10 mM MOPS buffer (pH 7.0) containing 5 μ M PQQ and 1 mM CaCl_2 at 4°C at least 30 min, washed with 10 mM MOPS (pH 7.0), and stored at 4°C until use.

PQQGDH/B-cytochrome *c* co-immobilized carbon paste electrode was immersed in 10 mM MOPS buffer, pH 7.0, containing 10 mM potassium ferricyanide or 1 mM mPMS.

Electrochemical Measurement

An Ag/AgCl electrode (Model RE-1, BAS Inc.) and Pt wire were used as reference and counter electrodes, respectively. The enzyme electrode (3 mm diameter, BAS Inc.), reference electrode, and counter electrode were joined to a 10-mL water-jacket cell (BAS Inc. Model VC-2) through holes in its Teflon cover. The potential was controlled by a potentiostat (HOKUTO-DENKO, Tokyo, Japan) in a three-electrode cell and currents were recorded with a recorder (Ohkura electric company, Tokyo, Japan). All measurements were carried out at 25°C in 10 mL of 10 mM MOPS buffer (pH 7.0) solution that was stirred at 250 rpm with a magnetic stirrer. The applied potentials were +400 mV and 100 mV vs. Ag/AgCl for the sensor employing potassium ferricyanide m-PMS, respectively. The measurements were carried out with the consecutive injections of 20 μ L of glucose solution in the reaction cell.

RESULTS

PQQGDH-B/Cytochrome *c* Co-immobilized Electrode

The PQQGDH-B-cytochrome *c* co-immobilized electrode, with potassium ferricyanide as the electrode mediator, responded immediately after injection of glucose samples and reacted steady state within 10 s (Fig. 3). In contrast, the enzyme electrode employing the same quantity of PQQGDH-B but without cytochrome *c* gradually increased the catalytic current, and reached the steady state after 1 min.

Figure 4 shows the correlations between current increase and glucose concentration for the sensors employing PQQGDH-B either or alone, with BSA, or with cytochrome *c*, using potassium ferricyanide as the electron mediator. The electrode with PQQGDH-B and cytochrome *c* co-immobilized at a ratio of 1:1000 showed about 50 times greater current



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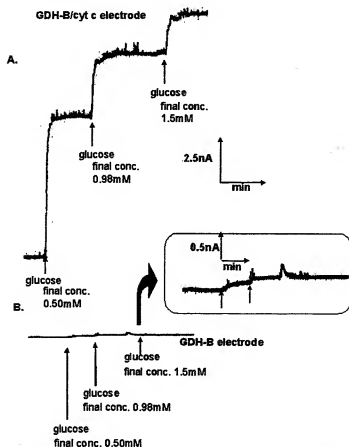


Figure 3. Response curve of the sensor with or without cytochrome *c*. A. PQQGDH/cytochrome *c* electrode (PQQGDH-B: cytochrome *c* molar ratio = 1:1000); B. PQQGDH-B electrode.

responses for glucose than with the electrode employing PQQGDH-B alone. For example, the sensor signal toward 20 mM glucose was 38 nA for the cytochrome *c* co-immobilized sensor, while the signal from the sensor immobilizing only PQQGDH-B was 0.75 nA. The co-immobilization of BSA with PQQGDH did not affect the sensor responses. Figure 5 shows the dose effect of the cytochrome for the current response. With the increase of the ratio of



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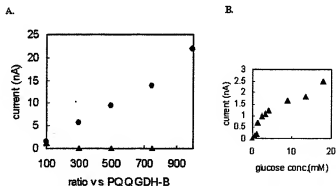


Figure 4. A. Correlation between current response and the molar ratio of PQQGDH/cytochrome *c* or PQQGDH/BSA. B. Calibration curve of PQQGDH-B/BSA electrode (the same mass of BSA was immobilized as cytochrome *c* at a molar ratio 100:1 vs. GDH). Closed circle; GDH electrode co-immobilized with various molar ratios of cytochrome *c*: GDH, closed triangle; GDH electrode co-immobilized with BSA instead of cytochrome *c*.

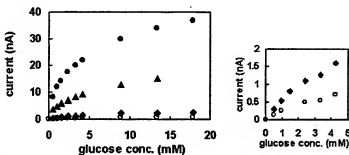


Figure 5. Electrode with different molar ratios of calibration curve of PQQGDH/cytochrome *c*: 1:1000 (closed circles), 1:500 (closed triangles), 1:100 (closed squares), and GDH alone (open circles). Reaction condition: 10 mM MOPS buffer pH 7.0, 10 mM Potassium ferricyanide, +400 mV vs. Ag/AgCl.

cytochrome *c* against the amount of PQQGDH immobilized in the electrode, the sensor signal increased, although no effect was observed with the increase of the content of BSA. These results clearly indicate that the presence of cytochrome *c* in PQQGDH, employing mediator type electrode, greatly facilitates the sensor signals.



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PQQGDH-B/Cytochrome b_{562} Co-immobilized Electrode

Figure 6 shows the correlations between the ferricyanide-based sensor response and glucose concentration of the enzyme electrode employing PQQGDH-B with and without cytochrome b_{562} . The co-immobilized enzyme electrode showed a more than 50-fold current increase compared with the electrode immobilized with only PQQGDH-B. For example, the sensor signal toward 20 mM glucose was 47 nA for the sensor co-immobilizing cytochrome b_{562} at a ratio of 1:100, while the sensor immobilizing only PQQGDH-B was 0.75 nA. The PQQGDH-B to cytochrome b_{562} ratio greatly influenced the sensor response, with the signal increasing as the ratio increases. Comparison of the results of Figs. 5 and 6 shows that cytochrome b_{562} is much more efficient than cytochrome c at improving the sensor signals. In order to achieve the same sensor response, over 10-fold more cytochrome c molecules are required than for cytochrome b_{562} .

Because mPMS is known to be an empirically better electron acceptor for PQQGDH than ferricyanide, we investigated the impact of the co-immobilization of cytochrome b_{562} and PQQGDH-B on the sensor employing mPMS as the electron mediator. The cytochrome b_{562} co-immobilization at a ratio of 1:100 (GDH:cytochrome b_{562}) resulted in a great improvement in sensor response (Fig. 7). The co-immobilization of cytochrome b_{562} with PQQGDH-B also resulted in the significant improvement in the sensor response with the enzyme electrode using mPMS as the electron mediator (Fig. 7). These results indicate that cytochrome can be utilized to improve the mediator dependent sensor response. Moreover, for the PQQGDH-B

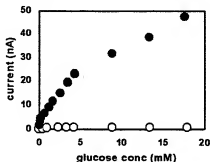


Figure 6. Calibration curve of PQQGDH-B/cytochrome b_{562} electrode. GDH electrode immobilized with a 100 fold molar excess of cytochrome b_{562} (closed circle) or without (open circle). Reaction condition: 10 mM MOPS buffer, pH 7.0, 10 mM potassium ferricyanide, +400 mV vs. Ag/AgCl.

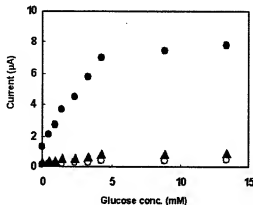


Figure 7. Calibration curve of PQQGDH-B/cytochrome electrode using mPMS as the mediator. GDH electrode was co-immobilized with a 100-fold molar excess of cytochrome *b*₅₆₂ (closed circles), or a 1000-fold molar excess GDH cytochrome *c* (closed triangles), or immobilized alone (open circles).

based enzyme electrode, cytochrome *b*₅₆₂ appears to have much superior characteristics than cytochrome *c*.

DISCUSSION

In this paper, we demonstrated that the addition of cytochromes as an interface molecule to facilitate the electron transfer between the enzyme and electron mediators improved the sensor response employing PQQGDH-B. In addition, we showed the superiority of the application of cytochrome *b*₅₆₂ from *E. coli* B as the electron transfer facilitator over that of cytochrome *c*.

Figures 5 and 6 clearly demonstrate that the GDH electrode shows a much greater current response when co-immobilized with cytochromes. Since the co-immobilization of BSA, instead of cytochrome, did not achieve the improvement of the sensor signals, the improvement of the current response was not due to the stabilization of enzyme during the enzyme electrode preparation process. We believe the cytochromes improved the sensor response by acting as the electron transfer protein between enzyme bound PQQ (catalytic center of PQQGDH) and the electron mediator (Fig. 8). Jin et al. reported that excess amount of free PQQ functioned as a redox shuttle between PQQGDH and cytochromes.^[31] In our



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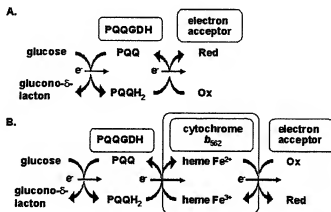


Figure 8. The proposed electron transfer pathway of GDH/*b*₅₆₂ electrode. Electron transfer pathways are shown for the electrodes with PQQGDH immobilized alone (A) and for that with PQQGDH and cytochrome co-immobilized (B).

experimental set-up, we did not add any free PQQ to the reaction chamber. Moreover, injection of glucose failed to produce a response in the absence of ferricyanide or mPMS as the electron mediator, although enough potential was applied for the oxidation of any available reduced PQQ. Therefore, the presence and contribution of free PQQ as the redox shuttle is negligible in our system.

Cytochrome *c* and cytochrome *b*₅₆₂ have similar redox potential (cytochrome *b*₅₆₂: +180 mV vs. SCE, cytochrome *c*: +250 mV vs. SCE). The difference in the contribution of cytochrome *b*₅₆₂ and cytochrome *c* to the response signal might be due to the differences in their accessibility to PQQ. Since the heme edge of cytochrome *b*₅₆₂ is more exposed compared with that of cytochrome *c*, the exposure of heme edge of cytochrome *b*₅₆₂ may contribute the adjacent of both redox centers of enzyme and cytochrome.

Because cytochrome *b*₅₆₂ is soluble and its production can be easily achieved in *E. coli*, cytochrome *b*₅₆₂ will be further utilized as an interface molecule to facilitate electron transfer. Further improvement of the affinity of cytochrome *b*₅₆₂ toward PQQGDH-B may lead to develop an electron transfer interface molecule for PQQGDH-B. This study demonstrated the future development of cytochromes as the essential component for the improvement of sensor response in the redox-enzyme based amperometric enzyme sensors.



REFERENCES

1. Anthony, C.; Turner, A.P.; Karube, I.; Wilson, G.S. *Biosensors—Fundamental and Applications*; Oxford University Press: 1987.
2. Linke, B.; Kerner, W.; Kiwit, M.; Pishko, M.; Heller, A. Amperometric Biosensor for in Vivo Glucose Sensing Based on Glucose Oxidase Immobilized in a Redox Hydrogel. *Biosens. Bioelectron.* **1994**, *9*(2), 151–158.
3. Laurinavicius, V.; Kurtinaitiene, B.; Liauksmas, V.; Jankauskaite, A.; Simkus, R.; Meskys, R.; Boguslavsky, L.; Skothiem, T.; Tanwnbaum, S. Reagentless Biosensor Based on PQQ-Depended Glucose Dehydrogenase and Partially Hydrolyzed Polyarbutin. *Talanta* **2000**, *52*, 485–493.
4. Tang, Z.; Louie, R.; Lee, J.H.; Lee, D.M.; Miller, E.E.; Kost, G.J. Oxygen Effects on Glucose Meter Measurements with Glucose Dehydrogenase- and Oxidase-Based Test Strips for Point-of-Care Testing. *Crit. Care. Med.* **2001**, *29*(5), 1062–1070.
5. Cleaton-Jansen, A.M.; Goosen, N.; Fayet, O.; Putte, P. Cloning, Mapping, and Sequencing of the Gene Encoding *Escherichia coli* Quinoprotein Glucose Dehydrogenase. *J. Bacteriol.* **1990**, *172*(11), 6308–6315.
6. Cleaton-Jansen, A.M.; Goosen, N.; Vink, K.; Putte, P. Cloning, Characterization and DNA Sequencing of the Gene Encoding the Mr 50,000 Quinoprotein Glucose Dehydrogenase from *Acinetobacter calcoaceticus*. *Mol. Gen. Genet.* **1989**, *217*(2–3), 430–436.
7. Sode, K.; Kojima, K. Improved Substrate Specificity and Dynamic Range for Glucose Measurement of *E. coli* PQQ Glucose Dehydrogenase by Site Directed Mutagenesis. *Biotechnol. Lett.* **1997**, *19*(11), 1073–1077.
8. Arief B. Witarto; Ohtera, T.; Sode, K. Site Directed Mutagenesis Study of Thermostable Chimeric PQQ Glucose Dehydrogenase and Its Structural Interpretation. *Appl. Biochem. Biotechnol.* **1999**, *77–79*, 159–168.
9. Yoshida, H.; Kojima, K.; Arief Budi Witarto; Sode, K. Engineering a Chimeric Pyrroloquinoline Quinone Glucose Dehydrogenase: Improvement of EDTA Tolerance, Thermal Stability and Substrate Specificity. *Protein Eng.* **1999**, *12*, 63–70.
10. Sode, K.; Shirahane, M.; Yoshida, H. Construction and Characterization of Linked-dimeric Pyrroloquinoline Quinone Glucose Dehydrogenase. L-GDH. *Biotechnol. Lett.* **1999**, *21*(8), 707–710.
11. Igarashi, S.; Ohtera, T.; Yoshida, H.; Arief B. Witarto; Sode, K. Construction and Characterization of Mutant Water Soluble PQQ Glucose Dehydrogenase with Altered Km Value—Site Directed



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- Mutagenesis Studies on the Putative Active Site. *Biochem. Biophys. Res. Commun.* **1999**, *264*, 820-824.
12. Sode, K.; Ohtera, T.; Shirahanae, M.; Arief B. Witarto; Igarashi, S.; Yoshida, H. Increasing the Thermal Stability of the Water-soluble Pyrroloquinoline Quinone Glucose Dehydrogenase by Single Amino Acid Replacement. *Enz. Microbiol. Technol.* **2000**, *26*, 491-496.
 13. Yamazaki, T.; Kojima, K.; Sode, K. Extended Range Glucose Sensor Employing Engineered Glucose Dehydrogenases. *Anal. Chem.* **2000**, *72*(19), 4689-4693.
 14. Kojima, K.; Arief B. Witarto; Sode, K. The Production of Soluble Pyrroloquinoline Quinone Glucose Dehydrogenase by *Klebsiella pneumoniae*—The Alternative host of PQQ Enzymes. *Biotechnol. Lett.* **2000**, *22*, 1343-1347.
 15. Yoshida, H.; Iguchi, T.; Sode, K. Construction of Multi-Chimeric Pyrroloquinoline Quinone Glucose Dehydrogenase with Improved Enzymatic Properties and Application in Glucose Monitoring. *Biotechnol. Lett.* **2000**, *22*, 1505-1510.
 16. Takahashi, Y.; Igarashi, S.; Nakazawa, Y.; Tsugawa, W.; Sode, K. Construction and Characterization of Glucose Enzyme Sensor Employing Engineered Water Soluble PQQ Glucose Dehydrogenase with Improved Thermal Stability. *Electrochemistry* **2000**, *68*(11), 907-911.
 17. Okuda, J.; Yoshida, H.; Kojima, K.; Himi, M.; Sode, K. The Role of Conserved His775/781 in Membrane-Binding PQQ Glucose Dehydrogenase of *Escherichia coli* and *Acinetobacter calcoaceticus*. *J. Biochem. Mol. Biol. Biophys.* **2000**, *4*, 415-422.
 18. Yoshida, H.; Araki, N.; Tomisaka, A.; Sode, K. *Enz. Microb. Tech.* **2001**, in press.
 19. D'Costa, E.J.; Higgins, I.J.; Turner, A.P. Quinoprotein Glucose Dehydrogenase and Its Application in an Amperometric Glucose Sensor. *Biosensors* **1986**, *2*(2), 71-87.
 20. Ohara, T.J.; Rajagopalan, R.; Heller, A. "Wired" Enzyme Electrodes for Amperometric Determination of Glucose or Lactate in the Presence of Interfering Substances. *Anal. Chem.* **1994**, *66*(15), 2451-2457.
 21. Matsushita, K.; Shinagawa, E.; Adachi, O.; Ameyama, M. Reactivity with Ubiquinone of Quinoprotein D-Glucose Dehydrogenase from *Glucobacter suboxydans*. *J. Biochem.* **1989**, *105*(4), 633-637.
 22. Fridman, V.; Wollenberger, U.; Bogdanovskaya, V.; Lisdar, F.; Ruzgas, T.; Lindgren, A.; Gorton, L.; Scheller, F.W. Electrochemical Investigation of Cellobiose Oxidation by Cellobiose Dehydrogenase in the Presence of Cytochrome *c* as Mediator. *Biochem. Soc. Trans.* **2000**, *28*(2), 63-70.



23. Habermüller, K.; Mosbach, M.; Schuhmann, W. Electron-Transfer Mechanism in Amperometric Biosensors. *Frenius J. Anal. Chem.* **2000**, *366*, 560–568.
24. Itagaki, E.; Hager, L.P. Studies on Cytochrome *b*₅₆₂ of *Escherichia coli*. I. Purification and crystallization of Cytochrome *b*₅₆₂. *J. Biol. Chem.* **1966**, *241*(16), 3687–3695.
25. Nikkila, H.; Gennis, R.B.; Sligar, S.G. Cloning and Expression of the Gene Encoding the Soluble Cytochrome *b*₅₆₂ of *Escherichia coli*. *Eur. J. Biochem.* **1991**, *202*(2), 309–313.
26. Moore, G.R.; Williams, R.J.; Peterson, J.; Thomson, A.J.; Mathews, F.S. A Spectroscopic Investigation of the Structure and Redox Properties of *Escherichia coli* Cytochrome *b*-562. *Biochim. Biophys. Acta* **1985**, *829*(1), 83–96.
27. Barker, P.D.; Nerou, E.P.; Freund, S.M.; Fearnley, I.M. Conversion of Cytochrome *b*₅₆₂ to *c*-type Cytochromes. *Biochemistry* **1995**, *34*(46), 15191–15203.
28. Feng, Y.; Sligar, S.G.; Wand, A.J. Solution Structure of Apocytochrome *b*₅₆₂. *Nat. Struct. Biol.* **1994**, *1*(1), 30–35.
29. Hamada, K.; Bethge, P.H.; Mathews, F.S. Refined Structure of Cytochrome *b*₅₆₂ from *Escherichia coli* at 1.4 Å Resolution. *J. Mol. Biol.* **1995**, *247*(3), 947–962.
30. Trower, M.K. PCR Cloning, Sequence Analysis and Expression of the *cybC* Genes Encoding Soluble Cytochrome *b*-562 from *Escherichia coli* B strain OP7 and K Strain NM522. *Biochim. Biophys. Acta* **1993**, *1143*(1), 109–111.
31. Jin, W.; Wollenberger, U.; Scheller, F.W. PQ as Redox Shuttle for Quinoprotein Glucose Dehydrogenase. *Biol. Chem.* **1998**, *379*(8–9), 1207–1211.

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